THE RESPIRATORY EXCHANGE OF DIAPAUSING PUPAE OF THE CECROPIA SILKWORM IN THE PRESENCE OF CARBON^{14} MONOXIDE [1]

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The inhibition of cytochrome oxidase by carbon monoxide stems from the remarkable similarity of this gas to oxygen. Recent work has shown that this similarity is even more far-reaching than formerly suspected, and that the combination of carbon monoxide with cytochrome oxidase may lead to phenomena in addition to a simple inhibition of respiration. For example, carbon monoxide may actually stimulate respiration (Daly, 1954). It may be combusted by oxygen, either enzymatically (Breckenridge, 1953), or catalytically; it may combine with water or alkali to produce formic acid or formates (Warburg, 1927).

The combustion of carbon monoxide has been studied by several workers including Clark, Stannard and Fenn (1950) and Black and Tyler (1959a, 1959b). The last-mentioned workers present a useful summary of the recent work on the oxidation of carbon monoxide, while the larger subject of the effects of carbon monoxide on living organisms has been ably reviewed by Lilienthal (1950).

Since the combustion of carbon monoxide may be superimposed on either the stimulation or the inhibition of respiration by carbon monoxide, the combustion of this gas has significant ramifications in studies of the carbon monoxide-inhibition of respiration. With present techniques for the measurement of radioactivity and for the analysis of small samples of respiratory gases, it is now feasible to disentangle the combustion of carbon monoxide from its effects on respiration.

The study reported here has combined these techniques to re-examine the respiratory exchange of diapausing pupae of the Cecropia silkworm in the presence of carbon monoxide and oxygen. A complete analysis has been achieved; namely, the simultaneous measurement of oxygen uptake, total gas uptake, and the production of $C^{14}O_2$ from $C^{14}O$. The results make possible a clear understanding of the complicated effects of carbon monoxide on the metabolism of diapausing pupae; they also provide new insight into the reaction of cytochrome oxidase with oxygen.

Materials and Methods

1. *Experimental animals*

The experiments were performed on diapausing pupae of the Cecropia silkworm which were reared under nets on wild cherry trees and managed as described

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by Shappirio and Williams (1957). Certain pupae were used during the five-month period of stable diapause (Williams, 1956); others were placed in permanent diapause by the removal of their brains (Williams, 1946).

In all experiments using radioactive carbon monoxide, the most anterior abdominal spiracles were cannulated with a short section of 0.011-inch bore polyethylene tubing (Clay-Adams) to eliminate the intermittent release of carbon dioxide described by Schneiderman and Williams (1955) and Buck and Keister (1955). Since the cannulation sometimes resulted in minor integumentary injury, the pupae were routinely stored at 25° C. for at least a month prior to use.

During the CO-combustion experiments the pupae were maintained in the dark with occasional exposure to dim light. Since the cuticle of the pupae is quite opaque the experiments may be viewed as having been conducted in the dark.

2. *Manometric measurements of gas uptake*

The rate of uptake of oxygen (Q_{O_2}) by pupae in air at 25° C. was determined by a modification of Warburg's "direct method" (Schneiderman and Williams, 1953). For the purpose of absorbing carbon dioxide, two filter papers moistened with a total of 1.6 ml. of 1 N potassium hydroxide were sealed in each vessel. The Q_{O_2} was expressed as the microliters of oxygen consumed per gram of initial live weight per hour. The initial live weight is the weight of the pupa within two months after pupation.

The measurements of carbon monoxide uptake reported in Section 2 of the Results and of total gas uptake in CO-O_2 mixtures at atmospheric pressure, reported in Sections 1 and 8 of the Results, were performed by the technique just described.

3. *Volumetric measurements of total gas uptake*

a) *At elevated pressures*

Measurements of total gas uptake at elevated pressures were performed at 25° C. in 45-ml. capillary volumeters by the use of the high pressure technique of Schneiderman and Feder (1954).

b) *At atmospheric pressure*

Except for the results reported in Sections 1 and 8, all measurements of total gas uptake in CO-O_2 mixtures were performed at 25° C. in simple 8- or 15-ml. capillary volumeters (Fenn, 1935) similar to the one described by Schneiderman and Feder (1954) but modified as shown in Figure 1 to permit the sampling of gases. The volumeter was assembled and flushed with 20 volumes of the experimental gas introduced through the vaccine stopper by means of a 22-gauge needle, and expelled *via* the capillary and attached rubber tubing. The experimental gas was trapped in the volumeter at atmospheric pressure by withdrawing the hypodermic needle and clamping off the rubber tubing. Finally, the index drop was tipped into the capillary and brought to rest at the desired point by removing the clamp on the rubber tubing and adjusting the gas volume with a 5-ml. syringe inserted through the vaccine stopper and filled with the experimental gas mixture.

In each experiment one volumeter served as a thermobarometer. The latter was assembled exactly as the animal-containing volumeters except that a glass "space-occupier," displacing 6.7 ml., was substituted for the pupa.

In addition to the thermobarometer, one to four animal vessels were fastened to a Lucite holder and placed in a constant temperature bath at 25.0° C. and allowed to equilibrate for one-half hour. Barium hydroxide was then injected into the sidearm *via* the vaccine stopper. Further details of procedure are included in Section 3 of the Results.

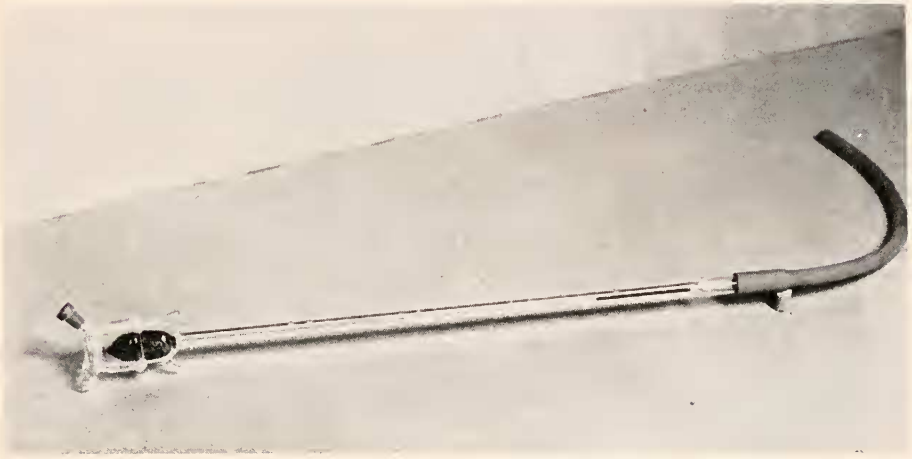

FIGURE 1. Volumeter used in the concomitant measurement at atmospheric pressure of total gas uptake, the oxygen uptake, and the production of carbon dioxide from carbon monoxide.

4. *Measurement of oxygen uptake in CO-O_2 mixtures by gas analysis*

Gas samples (ca. 1 ml.) were taken from each volumeter at the beginning and end of each experiment. The samples were analyzed for carbon dioxide and oxygen [3] in the Scholander half-cubic centimeter gas analyzer (Scholander, 1947), and the Q_{O_2} was calculated. The error introduced by the presence of carbon monoxide in the gas mixture was not in excess of 0.07% atm., as determined by analyses of carbon monoxide which had been purified as described below.

5. *Sampling and measurement of radioactive materials*

The $C^{14}O$ gas (New England Nuclear Corp.) was transferred by the method of Barton and Parrish (1956) as modified by Smetana (personal communication). Both $C^{14}O$-O_2 mixtures and $C^{14}O_2$ generated from $BaC^{14}O_3$ were counted in the gaseous phase in a Bernstein-Ballentine counting tube (Bernstein and Ballentine, 1950). The reproducibility in counting separate samples from the same gas mixture was in excess of 95%.

In certain experiments $BaC^{14}O_3$ was plated out on a millipore filter and counted as a solid on an automatic proportional flow counter (Baird Atomic; Model 750

[3] The gas analyses were performed by Mr. John Steen.

PF). When the radioactivity was less than 100 counts per minute, solid samples were counted on a windowless Geiger flow counter (Laboratory Associates, Inc.).

The volume of CO_2 that came from CO was calculated from the relationship:

$$\text{volume}_{CO_2} = \frac{\text{dpm}_{CO_2}}{\text{dpm}_{CO}} \times \text{volume}_{CO} = \frac{k\ \text{cpm}_{CO_2}}{k'\ \text{cpm}_{CO}} \times \text{volume}_{CO}$$

Provided both $C^{14}O$ and $C^{14}O_2$ are counted under identical conditions, the constants k and k′ will be equal and the volume of carbon monoxide combusted can be calculated directly.

6. *Experimental gases*

The carrier carbon monoxide was the Matheson product (C.P.) assaying 99.9% carbon monoxide. This gas was passed through three serially arranged wash bottles, each equipped with a sintered glass dispersion element. The first bottle contained 10% potassium hydroxide to remove any residual carbon dioxide and carbonyl compounds; the second contained a saturated solution of lead acetate to remove any sulfur compounds; and the third vessel contained distilled water to saturate the gas with water vapor.

U.S.P. grade oxygen and U.S.P. grade carbon dioxide, each assaying 99.5%, were obtained from New England Gas Products, Inc. Since the major impurities in these gases were nitrogen, argon, oxygen (0.08% in the CO_2), and H_2O, no further purification was made.

Gas mixtures were prepared at atmospheric pressure and room temperature in a 6-liter, water-sealed spirometer. The gas composition was determined accurately with the Scholander gas analyzer (Scholander, 1947) immediately before use.

Experimental Results

1. *Manometric evidence for the combustion of carbon monoxide*

In manometric experiments there is no quantitative way to distinguish between gas disappearance due to the combustion of carbon monoxide and that due to respiration. However, it is relatively easy to discover in a qualitative sense whether the combustion of carbon monoxide is going on. It will be recalled that this combustion involves the disappearance of one molecule of oxygen for every two molecules of carbon monoxide that are burned. A known volume of oxygen and an excess of carbon monoxide can be sealed in a Warburg flask, together with an animal and alkali. If all of the oxygen is removed by carbon monoxide-combustion, the amount of gas disappearing from the flask will be three times the known amount of oxygen present; if all of the oxygen is removed by respiration, the amount of gas disappearing will be equal to the amount of oxygen present; and finally, if part of the oxygen is removed by carbon monoxide-combustion and part by respiration, the total amount of gas disappearing will assume an intermediate value (Thimann *et al.*, 1954).

Six preparations of this type were assembled. In each preparation a diapausing pupa was sealed in a 45-ml. vessel together with a filter paper moistened with 1.6 ml. of 1 N potassium hydroxide. The vessels were each flushed with a slowly

flowing mixture of 99% atm. carbon monoxide and 1% atm. oxygen for 15 hours and then sealed. The total amount of oxygen initially present in each animal vessel was therefore 1% of the gas volume of the sealed system. A seventh air-filled vessel served as thermobarometer.

The time course of total gas uptake in a typical preparation, corrected for the reaction of carbon monoxide with alkali as described below, is plotted in Figure 2. The amount of oxygen present at the outset is indicated on the graph. It will

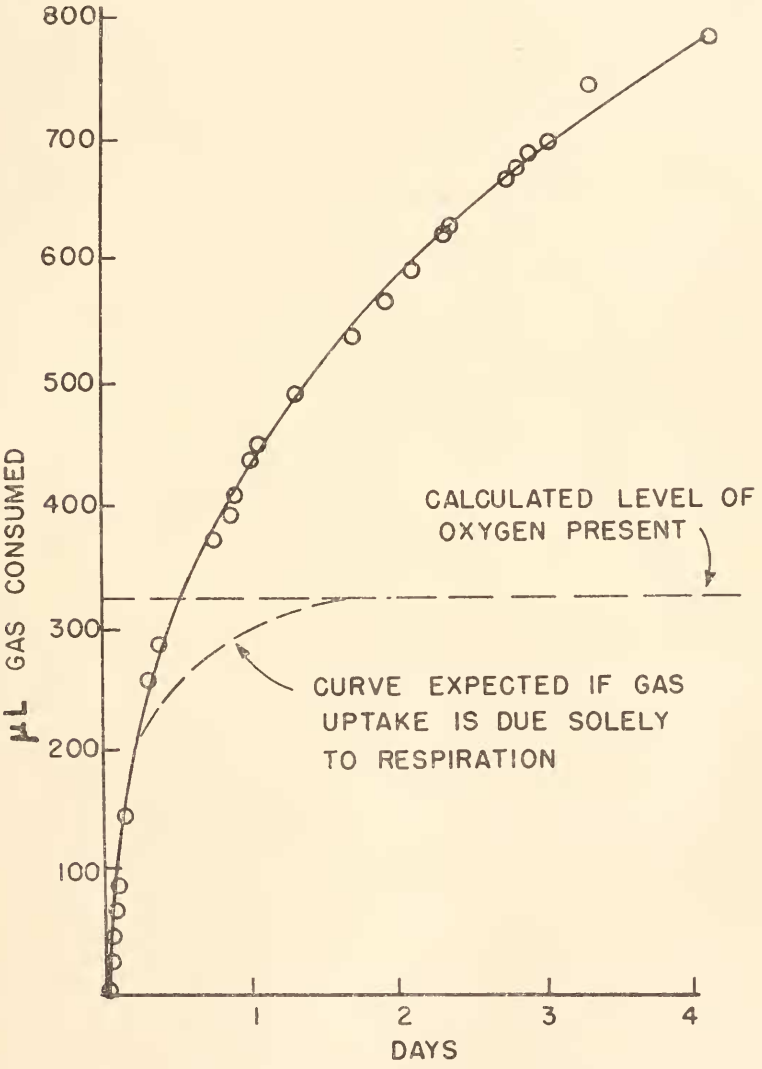

FIGURE 2. The time-course of the disappearance of gases from a sealed Warburg vessel containing a diapausing pupa in the presence of alkali and a gas mixture of 99% atm. carbon monoxide and 1% atm. oxygen. Each datum has been corrected for the reaction of carbon monoxide with the alkali.

be observed that gas continued to disappear long after the calculated 325 μl. of oxygen would have been used up. The substantial anaerobic capacity of the pupae (Harvey and Williams, 1958a; 1958b) enabled the continuation of the experiment for four days. At that time the function was tending toward a plateau somewhat short of the 975 μl. (325 μl. of oxygen plus 650 μl. of carbon monoxide) predicted for removal of the oxygen solely by carbon monoxide-oxidation. These results, together with the similar results obtained with the other five preparations,

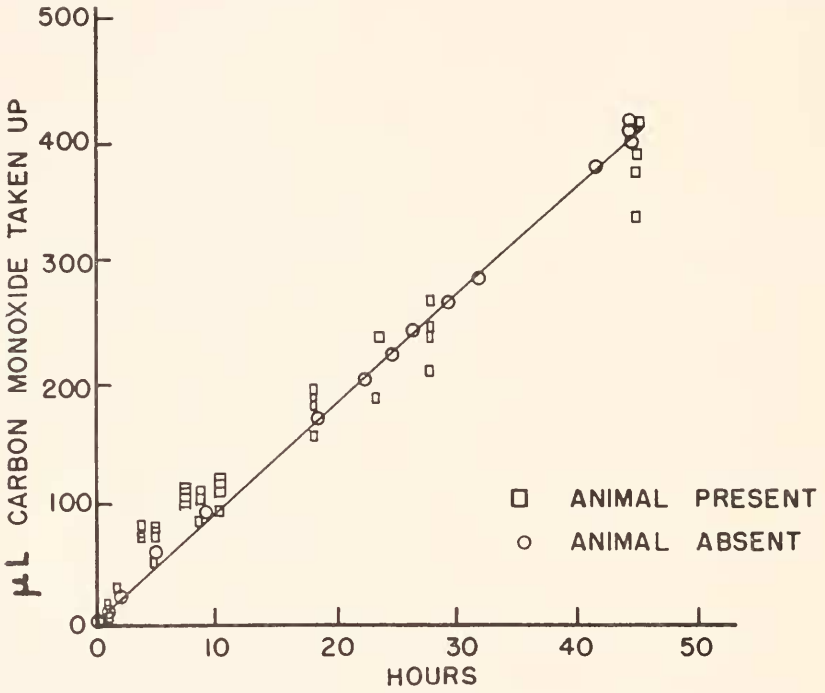

FIGURE 3. The time-course of the disappearance of carbon monoxide from Warburg vessels containing alkali and carbon monoxide, but no oxygen.

clearly demonstrate that carbon monoxide is removed from the system by the living pupa. Since this does not occur in the absence of oxygen (see Section 2) one may infer that carbon monoxide is being combusted.

2. Correction for the non-oxidative removal of carbon monoxide

The manometric data just described were corrected for the reaction of carbon monoxide with alkali (Warburg, 1927) in the following way. Four 45-ml. Warburg vessels were prepared as in the above experiment but no animals were added. Three were flushed with 20 volumes of carbon monoxide that had been washed through potassium hydroxide and lead acetate (Method 6). The fourth vessel contained air and served as a thermobarometer. The uptake of carbon monoxide from each vessel, plotted as a function of time in Figure 3, amounted to 9.2 ± 0.2 (S.D.) μl./hour.

An essentially similar experiment was performed to determine whether the living pupa catalyzed any further non-oxidative uptake of carbon monoxide. When a pupa was added to the system the rate of carbon monoxide uptake was identical with that in the absence of the pupa (Fig. 3). Therefore, in the oxygen-free system there is no indication that the pupa catalyzes any further non-oxidative uptake of carbon monoxide.

The empirical correction factor for the reaction of carbon monoxide with alkali was subtracted from the gas disappearance in the experiment reported in Section 1. The absence of non-oxidative uptake of carbon monoxide by the diapausing pupa enabled an alternative method of correction in subsequent experiments. The thermobarometer was simply flushed with the experimental gas mixture . . . the thermobarometer correction then automatically compensated for the reaction of carbon monoxide with alkali.

In summary, we can state that the rate of gas disappearance in CO-O_2 mixtures is composed of a component due to the reaction of carbon monoxide with alkali, a component due to the combustion of carbon monoxide, and a component attributable to the respiration of the pupa. The experiment reported in the following section was designed to disentangle these latter two phenomena.

3. *Special technique for the concomitant determination of total gas uptake, oxygen uptake and production of carbon dioxide from carbon monoxide*

The total gas uptake (Q_{total}) of a pupa is equal to the oxygen uptake due to respiration ($Q_{O_2}R$), plus the oxygen uptake due to CO-combustion ($Q_{O_2}C$), plus the carbon monoxide uptake due to CO-combustion (Q_{CO}). The latter value is numerically equal to the CO_2 production due to CO combustion ($Q_{CO_2}C$).

$$Q_{total} = Q_{O_2}R + Q_{O_2}C + Q_{CO_2}C$$

From this relationship it is immediately clear that measurements of the total gas uptake and the oxygen uptake due to respiration and combustion combined allow us to calculate the amount of carbon dioxide production due to CO-combustion. In addition, this latter value can be determined directly using carbon^{14}-labeled carbon monoxide.

Each of four diapausing pupae was placed in a modified Warburg vessel and its oxygen uptake in air measured manometrically (Method 2). The pupae were then removed carefully from the Warburg vessels and each was sealed in a modified Fenn volumeter with a gas phase of 82% atm. CO and 18% atm. O_2, as described under Method 3b. In order to introduce a known amount of barium hydroxide, and of $C^{14}O$, and to remove samples for analysis of respiratory gases, the following techniques were employed in elaboration of the operations described in Method 3b.

The index drop of each volumeter was brought to the zero mark by withdrawing gas, using a hypodermic syringe equipped with a #25 blunted needle and lubricated with silicone grease. Then exactly 1.00 ml. of barium hydroxide (0.097 *M* $Ba(OH)_2 \cdot 8\ H_2O$) was added to the sidearm from a second 1-ml. syringe. Using a third 1-ml. syringe, approximately 1 ml of gas was removed for gas analysis, leaving the meniscus of the index drop at zero. Now 0.800 ml. of the same CO-O_2 mixture was added to each animal vessel and 0.500 ml. to the

thermobarometer. Finally 0.200 ml. of $C^{14}O$ (ca. 36 μc./ml.) was added to each vessel so that the meniscus on each animal-containing volumeter now read 1.000 ml. and that on the thermobarometer read 0.700 ml. The excursions of the index drops were read at intervals of from 1 to 5 hours for a total period of 16 hours. During this same period three 0.200-ml. samples of gas were withdrawn from each volumeter to measure the specific activity of the $C^{14}O$ in the gas phase. As the pupa consumed gas the index drop moved toward zero. Pure oxygen was injected at convenient intervals to return the index drop to 1.000 and to restore the gas phase to its initial composition.

Finally, at the end of the experiment the index drops were all brought to zero by withdrawing gas; a second 1.000 ml. of barium hydroxide was added and the vessels shaken thoroughly to remove the last traces of unabsorbed CO_2. A final 1-ml. sample of gas was taken for analysis of respiratory gases. The vessels were

TABLE I

Comparison in normal diapausing pupae of the rate of oxygen uptake in air with the total gas uptake, oxygen uptake, and $C^{14}O_2$ production in a mixture of CO and O_2*

(a) Animal #	(b) In air Q_{O_2}**	In CO-O_2 mixture*			(f) Per cent of total uptake in CO-O_2 that is attributable to CO uptake
		(c) Q_{total}**	(d) Q_{O_2}**	(e) $Q_{C^{14}O_2}$**	
1085	13	22.7	22.0	1.38	6.1
1086	15	27.1	23.1	1.10	4.1
1087	11	23.7	21.6	1.57	6.6
1088	11	23.1	22.2	0.91	3.9
Average	12	24.1	22.2	1.24	5.2

* The gas mixture was 82% atm. CO and 18% atm. O_2.
** Q values in μl. per gram initial live weight per hour.

removed from the bath and opened. The $BaCO_3$ was plated out on a millipore filter, oven-dried, and transferred to the gas-generating chamber on the manifold; the CO_2 was generated with concentrated H_2SO_4, introduced into the counting chamber and counted.

The rates of uptake of total gas, oxygen, and carbon[14] dioxide are recorded for each of four animals in Table I, together with the corresponding rate of oxygen uptake in air. These data are representative of the results obtained with 48 pupae in 12 experiments essentially similar to the one being described.

4. *The stimulation of respiration by carbon monoxide*

Attention is first directed to columns b, c, and d of Table I. The average Q_{O_2} *in air* was 12 μl./gram/hour (column b). In the presence *of the CO-O_2* mixture the Q_{total} was almost exactly twice this value (column c). However, the Q_{O_2} *in this mixture,* as determined by direct gas analysis (column d), was nearly as high as the Q_{total} in this mixture. Therefore, the stimulation of gas uptake in the presence of CO and O_2 is due almost entirely to an increase in the rate of the true respiration of the pupa. (It should be emphasized that the term "respiration"

refers to the normal metabolic process and does not include the uptake of carbon monoxide and oxygen associated with carbon monoxide-combustion.)

5. *The combustion of carbon monoxide to carbon dioxide*

Columns c and d of Table I show that in the case of each pupa the oxygen uptake in the CO-O_2 mixture is not quite as large as the total gas uptake. On the average the total gas uptake is about 2 μl./gram/hour greater than the average oxygen uptake. This discrepancy, together with the data reported in Section 1, suggests that carbon monoxide is being oxidatively removed from the vessels.

TABLE II

Calculation of total CO_2 that came from CO based on isotopic measurements

(a) Animal #	(b) *cpm per 0.200 ml. sample of original gas mixture**	(c) *cpm per 0.200 ml. of CO (calculated)	(d) *Total cpm in expired CO_2	(e) μl. of CO_2 that came from CO
Thermobarometer	112,000	92,000	−31	−0.068
1085	250,000	205,000	157,195	154
1086	147,000	121,000	54,324	101
1087	174,000	143,000	105,145	154
1088	53,400	43,800	19,466	89

* The values recorded have all been corrected for background activity (*ca* 500 cpm).
** The gas mixture was 82% atm. CO and 18% atm. O_2.

This conclusion is proven beyond reasonable doubt by the results recorded in column e. It is clear that $C^{14}O_2$ is being produced from $C^{14}O$ during the experiment at a rate of about $1.2 \pm 10\%$ μl./gram/hour. This amount of gas, together with the oxygen uptake (from gas analysis), accounts completely for the total gas uptake in the CO-O_2 mixture.

These data are presented in further detail in Table II. The background level of radioactivity in the $BaCO_3$ from the thermobarometer (column d) assures us that we are not dealing with a non-specific trapping of $C^{14}O$ in the alkali. Further isotopic experiments (Harvey and Smetana, unpublished observations) demonstrated that neither the catalytic effect of water on CO-combustion nor the solubility of $C^{14}O$ in the tissues of the pupae contributed in any way to the results reported in this paper.

6. *The fate of C^{14}-labeled compounds within the pupa*

a) *Under aerobic conditions*

At the end of the experiment described in Sections 3, 4 and 5, two pupae (nos. 1086 and 1087) were immediately desiccated under high vacuum for half a day. The gases that came from the pupae during this treatment were trapped in $Ba(OH)_2$. The $BaCO_3$ was treated with acid and the generated CO_2 was counted. The $C^{14}O_2$ given off in this way was added to that given off during the experiment and contributes to the $Q_C{}^{14}{}_{O_2}$ reported in column e of Table I.

The desiccated remains of the pupa were ground up and oven-dried. Two

TABLE III

The fate of carbon^{14}-labeled compounds formed within the pupa during exposure to $C^{14}O$ under aerobic and anaerobic conditions

Animal	Condition during experiment	$C^{14}O_2$ (μl.) given off during experiment	$C^{14}O_2$ (μl.) distilled from pupa after experiment	C^{14}* compounds retained by pupa after distillation	
				Non-acidified residue	Acidified residue
1086	aerobic	80	10	11	5.6
1087	aerobic	127	21	5.9	2.0
1094	anaerobic	7.4	0.14	<0.4	<0.4
1095	anaerobic	11.1	0.13	<0.4	<0.4

* Expressed as μl. of $C^{14}O$.

aliquots were taken—one was counted directly and the other was acidified with 6 N HCl, washed with water, re-dried and counted in the same counter. Expressed as μl. of $C^{14}O$, there was an average of 8 μl. trapped in the pupa as calculated from the untreated aliquot, and an average of 4 μl. fixed as calculated from the acidified aliquot (Table III). Thus the reactions of CO and CO_2 combined could have resulted at most in the fixation of 4 μl. of $C^{14}O$.

b) *Under anaerobic conditions*

It is possible that the fixation of $C^{14}O$ is masked by the immediate oxidation of the resulting carbonyl compound by some aerobic process. To favor the accumulation of carbonyl compounds the following procedure was employed.

Each of two cannulated, brainless diapausing pupae was sealed in a volumeter containing $Ba(OH)_2$ with a gas phase of pure CO that had been passed through Fieser's solution (Fieser, 1924) (to assure the complete absence of oxygen from the gas phase), lead acetate, and water. The pupae were allowed to equilibrate for seven hours so that they might use up the last traces of oxygen dissolved within their tissues (Harvey and Williams, 1958a). Then $C^{14}O$ was added to give a final specific activity of 1 million cpm/ml. After 18 further hours each pupa was removed and its gases distilled into $Ba(OH)_2$ as described in the preceding section. The residue of each pupa was dried and divided into an acidified and an un-acidified fraction, each of which was plated out and counted. Each of the four aliquots yielded the normal background count (for this counter) of 18 to 19 cpm. Since this method is sensitive enough to detect the fixation of 0.4 μl. of gas, it is clear that no more than this amount of $C^{14}O$ was fixed under anaerobic conditions (Table III).

c) *The discharge of $C^{14}O_2$ from pupae*

When the experiment described in Sections 3, 4 and 5 was terminated, each of two pupae (nos. 1085 and 1088) was placed in a sealed container with $Ba(OH)_2$. The $BaCO_3$ was collected during each of three intervals from each pupa. The CO_2 was generated with acid and counted. The rate of discharge of $C^{14}O_2$ during and after the experiment is plotted as a function of the time after the ex-

periment in Figure 4. It is clear that the rate of release of $C^{14}O_2$ declines rapidly and after 4 days almost all of the labeled gas has been expired. This $C^{14}O_2$ given off after the experiment was added to that collected during the experiment in the calculations of $Qc^{14}{}_{O_2}$ recorded for these pupae in Table I.

7. CO-combustion and the level of metabolism

It is possible to vary the level of metabolism in a diapausing pupa merely by rupturing the integument. Such injured pupae consume oxygen at rates several times that of uninjured individuals (Schneiderman and Williams, 1953). This

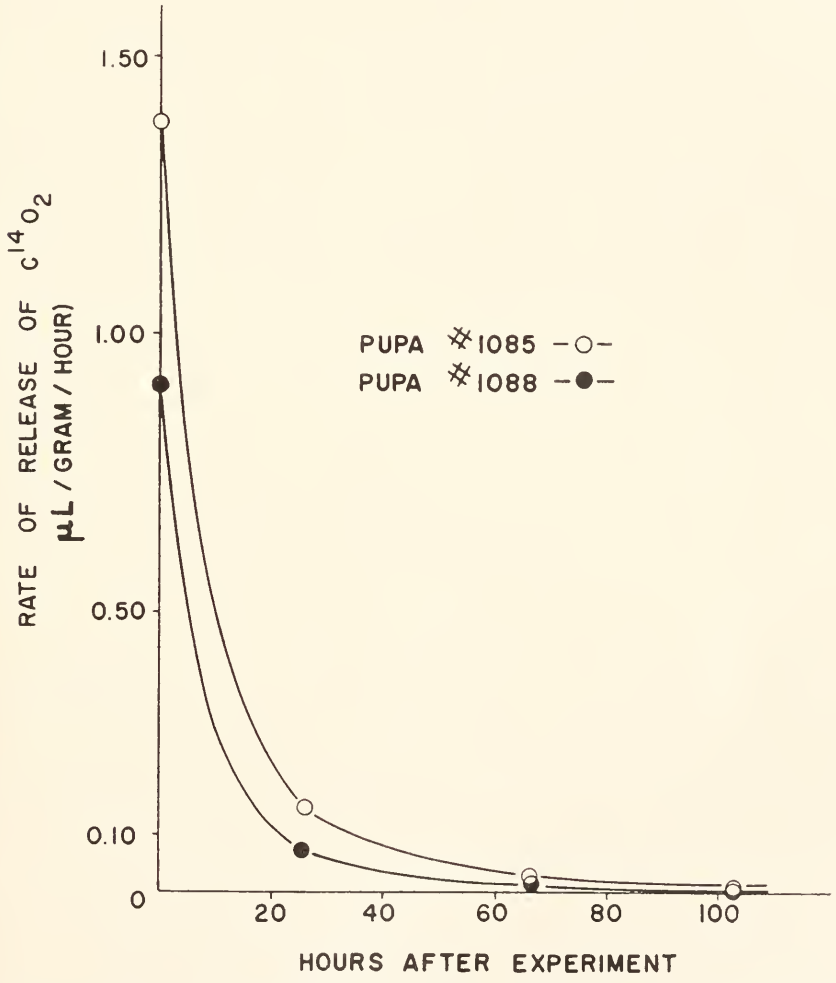

FIGURE 4. The rate of release of $C^{14}O_2$ from diapausing pupae, during exposure to a $C^{14}O$-O_2 mixture (plotted at 0 hours), and during the subsequent period after the exposure was terminated. It is seen that the rate of $C^{14}O_2$ expiration follows a decay function, and that by four days after the termination of exposure all of the radioactive gas has been expired.

TABLE IV

The rate of oxygen uptake of injured pupae in air compared with their total gas uptake and $C^{14}O_2$ production in a mixture of CO and O_2

Animal	In air Q_{O_2}**	In CO-O_2 mixture*		Per cent of total gas uptake that is attributable to CO-combustion
		Q_{total}**	$Q_{C^{14}O_2}$**	
943	76	91	3.22	3.5
944	26	37	1.12	3.0
963	36	38	1.41	3.7
Average	46	55	1.92	3.5

* The gas mixture was 82% atm. CO and 18% atm. O_2.
** Q values in μl. per gram initial live weight per hour.

phenomenon allows one to inquire into the relationship between metabolic rate and CO-combustion.

For this experiment each of three pupae was injured by the establishment of a terminal abdominal window (Williams, 1952). After a week the rate of oxygen uptake was measured in air, using the modified Warburg technique.

The pupae were then exposed to 82% atm. CO and 18% atm. O_2 exactly as described in the experiment in Section 3, except that no gas samples were withdrawn for gas analysis. The $C^{14}O_2$ given off during the experiment and during the 82 hours immediately following the experiment was plated out, generated with acid, and counted. In Table IV the Q_{O_2} in air, and the Q_{total} and the $Q_{C^{14}O_2}$ in the CO-O_2 mixture are presented.

It is clear from these somewhat preliminary data that carbon monoxide is combusted at a slightly higher rate in the injured pupa than in the uninjured pupa. There is some indication that there may be a linear increase in $Q_{C^{14}O_2}$ as a function of Q_{O_2} in air.

8. *The inhibition of the injury metabolism by carbon monoxide*

Having dealt quantitatively with the combustion of carbon monoxide in both normal and injured diapausing pupae, we are in a position to evaluate the effects of this gas on the true respiration of the pupa.

The rate of oxygen consumption of each of 24 brainless diapausing pupae was first measured in air (Method 2). The individuals were then divided into three groups and treated as follows. The rate of gas disappearance of each of nine pupae was re-determined in a CO-O_2 mixture of 90% atm. carbon monoxide and 10% atm. oxygen ($CO/O_2 = 9/1$); the rate for each of nine pupae in 93% atm. carbon monoxide and 7% atm. oxygen ($CO/O_2 = 13/1$); and (making use of the high pressure technique) the rate for each of six pupae, in 500% atm. carbon monoxide and 21% atm. oxygen ($CO/O_2 = 24/1$). The results in each case were corrected for the reaction of carbon monoxide with alkali but not for the combustion of CO to CO_2 (see Discussion (3) for correction for CO-combustion).

Similar determinations were made on a total of ten brainless diapausing pupae

which, one week previously, had been subjected to a large leg injury (Schneiderman and Williams, 1953).

The results are summarized in Figure 5 along with data from the pupae exposed to a mixture of 82% atm. carbon monoxide and 18% atm. oxygen ($CO/O_2 = 5/1$) described in Sections 3 and 5 of the Results. (For simplicity, the results in the CO-O_2 mixture of 13/1 are not presented in the Figure.)

The ratio between the rates of decrease in gas volume in CO-O_2 mixtures and in air have been computed for each animal and plotted as a function of the corre-

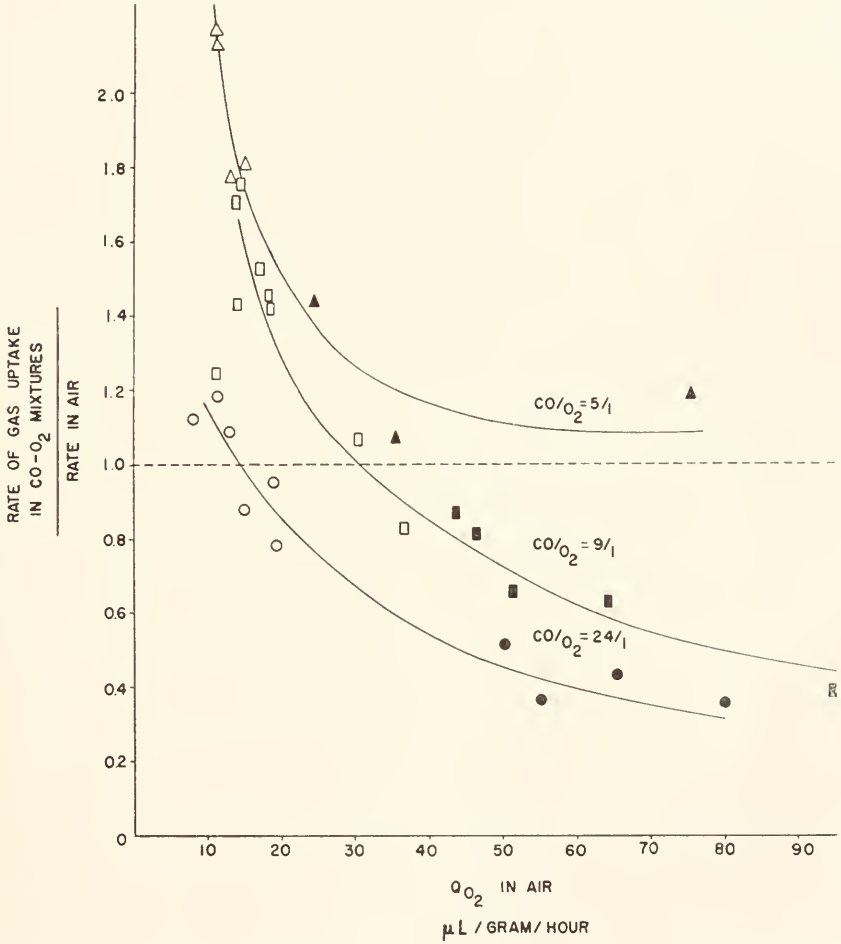

FIGURE 5. For a series of uninjured and injured pupae of the Cecropia silkworm, the relative rates of gas uptake in carbon monoxide *versus* air are plotted as a function of the Q_{O_2} of the corresponding animals in air. Each datum has been corrected for the reaction of carbon monoxide with alkali but not for carbon monoxide-combustion. All points above the dotted line signal an acceleration of gas uptake by carbon monoxide. Points below this line signal a depression by carbon monoxide. The open points are uninjured pupae; the solid points are injured pupae.

sponding Q_{O_2} in air. It will be observed that for animals exposed to the mixture of 82% atm. CO and 18% atm. O_2 this ratio is greater than 1, no matter what the prior Q_{O_2} in air. This means that the gas uptake is invariably higher in the presence of this CO-O_2 mixture than it is in air. When the amount of carbon monoxide in the mixture is increased (lower curves) it will be noted that this ratio remains greater than 1 only for pupae exhibiting a low Q_{O_2} in air. In pupae whose metabolism has been enhanced by injury, the ratio is invariably less than 1, indicating that the rate of gas uptake is inhibited. Finally in each of the lower curves there is an intermediate level of metabolism where carbon monoxide neither stimulates nor inhibits.

Discussion

1. *Carbon monoxide-combustion and cytochrome oxidase*

Most of the evidence in the literature suggests that the combustion of carbon monoxide by oxygen is mediated by cytochrome oxidase (Breckenridge, 1953; Black and Tyler, 1959b). The most damaging evidence for this hypothesis is the augmentation of CO-combustion in light (Stannard, 1940; Black and Tyler, 1959a) where, presumably, the cytochrome oxidase-carbon monoxide complex is very unstable. However, Black and Tyler (1959a) attempt with some success to account for this light-stimulation of CO-oxidation. Stannard (1940) and Chance (1949) have implied that the combustion of carbon monoxide is mediated by catalase. However, Breckenridge (1953) exposed several purified enzyme preparations, including catalase, cytochrome *c* and diaphorase, to mixtures of 80% atm. $C^{14}O$ and 20% atm. O_2. He showed that only cytochrome oxidase was able to cause the formation of $C^{14}O_2$; moreover, this activity was augmented as the enzyme was purified.

As a third alternative, Wald (1959) has proposed that an intermediate (RH) might be carbonylated to the corresponding aldehyde (RCOH). The latter might subsequently reduce water, yielding two hydrogens, while the aldehyde was being oxidized to the acid (RCOOH). The hydrogens would be available as substrate for the cytochrome system while the acid could be decarboxylated to yield carbon dioxide and thereby regenerate the intermediate (RH) which would thus act as a catalyst.

The results of the present study argue against the formation of a carbonylated intermediate. The failure to detect carbon^{14}-labeled material in the dried residue of the anaerobic pupa (Table III) argues that no direct fixation of $C^{14}O$ occurred. The remote possibility exists, however, that the small amount of $C^{14}O_2$ given off during the anaerobic experiment may have been the oxidation product of some carbonylated intermediate. Only a trace of carbon^{14}-labeled compound other than $C^{14}O_2$ was found in the aerobic experiment where as many as 127 μl. of this gas were formed (Table III). This small aerobic carbon^{14} fixation was undoubtedly due to the fixation of some endogenous $C^{14}O_2$ produced within the pupa. Finally, it will be recalled that there was no manometric evidence for carbon monoxide uptake by the anaerobic pupa (Fig. 3).

These findings fail to support Wald's theory of the carbonylated intermediate. Neither do they entirely disprove it, since the fixation of less than 0.4 μl. (9 μ

moles) of carbon monoxide could not be detected by this procedure. Possibly an accumulation of less than 9 μ moles of intermediate would be sufficient for Wald's carbonylation-catalytic process.

The simplest interpretation of these data is that both $C^{14}O$ and O_2 react directly with cytochrome oxidase to form $C^{14}O_2$. Some of the $C^{14}O_2$ is exhaled immediately as a gas, some goes into solution in the tissues, some exchanges with tissue bicarbonate, eventually to be given off as a gas, while presumably some of the $C^{14}O_2$ is fixed (Table III).

This interpretation would seem to require either that CO and O_2 react in sequence with cytochrome oxidase or, more simply, that they react simultaneously with this enzyme. This second prospect implies that there is more than one combining site on the cytochrome oxidase molecule. Wald and Allen (1957) find that, in the reaction of carbon monoxide with cytochrome oxidase, the coefficient n is greater than 1 in the Hill equation. This finding implies an interaction at the catalytic site between more than one molecule of carbon monoxide on cytochrome oxidase which, in turn, implies the presence of more than 1 heme per active molecule.

Ambe and Venkataraman (1959) report that in highly purified, homogeneous preparations of cytochrome oxidase there is but one heme per monomer. However, they also find one copper atom per heme group. Of the greatest importance was their finding that their monomer displayed no cytochrome oxidase activity unless phospholipide was added.

These seemingly contradictory observations are reconciled by the hypothesis that several individual units (monomers) of cytochrome oxidase are oriented on the phospholipide in such a way that interaction between two or more monomers is possible.

Although there are many other interpretations of these data, the combustion of carbon monoxide by molecular oxygen is a fact which must be accounted for, and an instrument which must not be overlooked, in efforts to understand the mechanism by which cytochrome oxidase activates oxygen.

2. *Carbon monoxide and the metabolism of diapausing pupae*

The diapausing pupa is particularly responsive to carbon monoxide. In no other animal known to us has CO-stimulation of respiration been clearly demonstrated.

CO-combustion, on the other hand, is well-known in a variety of animals. In Cecropia pupae this reaction accounts for but from 5 to 7% of the total gas uptake in CO-O_2 mixtures, in marked contrast to the 57% found in frog muscle (Clark, Stannard and Fenn, 1950). In developing sea urchin eggs the values range from 10 to 43% (Black and Tyler, 1959a). It is tempting to speculate that all aerobic cells are capable of catalyzing CO-oxidation and that the magnitude of the phenomenon varies from animal to animal depending perhaps, as Breckenridge (1953) has suggested, on the state of oxidation of their cytochrome oxidase.

3. *Carbon monoxide-inhibition of the true respiration of injured pupae*

When the gas uptake due to CO-combustion is subtracted from the total gas uptake, the true respiration in CO-O_2 mixtures is revealed. From Tables I and

IV we see that (with a $CO\text{-}O_2$ ratio of 5 to 1) we must subtract 1.8 μl./gram/hour for CO-combustion of normal diapausing pupae and 2.9 μl./gram/hour for injured pupae. Extensive semi-quantitative experiments (Harvey, unpublished) show that at higher $CO\text{-}O_2$ ratios the gas uptake due to CO-combustion is somewhat less, though still easily detectable with radioactive material. Since the correction is small compared to the total gas uptake of injured pupae, the values mentioned above are sufficiently accurate for our present purposes.

Thus the effects of $CO\text{-}O_2$ mixtures on the true respiration of pupae follow the same functions as those presented in Figure 5 except that each curve is shifted for from 0.2 to 0.4 units toward the abscissa. This means that the stimulation of true respiration by carbon monoxide is somewhat less than that indicated by considerations of the total gas uptake, and the inhibition of true respiration is somewhat greater.

This CO-inhibition of the true respiration of injured pupae is of considerable theoretical interest. Harvey (1956) reports that the total gas uptake of injured pupae is inhibited by carbon monoxide—a finding subsequently confirmed by Kurland and Schneiderman (1959). We can now state that the true respiration of injured pupae is inhibited to an even greater extent. Harvey and Shappirio (unpublished observations) have shown that this augmented CO-sensitivity of the injury-enhanced true respiration of pupae is attributable to an increase in the activity of certain cytochrome enzymes.

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Summary

1. After due correction for complicating reactions, the rate of total gas uptake of normal diapausing pupae of the Cecropia silkworm in a carbon monoxide-oxygen mixture of 5 to 1 is found to be stimulated approximately 2-fold over the rate in air.

2. Ninety per cent of this *extra* gas uptake is due to a stimulation of the true respiration of the pupa.

3. The remaining ten per cent is due to the combustion of carbon monoxide to carbon dioxide.

4. The combustion of carbon monoxide is increased slightly when the metabolism of the pupa is enhanced following integumentary injury.

5. There is a slight fixation of carbon-14 in pupae under aerobic conditions, presumably as a result of fixation of $C^{14}O_2$ manufactured from $C^{14}O$ by the pupa.

6. There is no detectable fixation of $C^{14}O$ in pupae exposed to this gas under anaerobic conditions.

7. After correction for carbon monoxide-combustion, it is clear that the enhanced oxygen uptake following integumentary injury of diapausing pupae is inhibited by carbon monoxide.

8. This carbon monoxide-inhibition is directly proportional to the enhancement of oxygen uptake following injury.

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